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JB Review

Roles of stromal microenvironment in colon cancer progression†

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Although our understanding of epithelial cancer cells has advanced significantly, our understanding of the cancer microenvironment is still fragmentary. In contrast to our intuitive impression that our body always suppresses cancer growth, recent pieces of evidence show that cancer often exploits our body reactions to expand, invade local tissues and metastasize to distant organs. Accordingly, investigations of such body reactions in the tumour microenvironment should help us to design novel therapeutic strategies that can be combined with the traditional therapeutics targeted at the cancer cells themselves. In this article, I am going to review our recent efforts in search of novel therapeutic strategies against colon cancer using mouse models.

Keywords: COX2/chemokines/invasion/metastasis/Notch signalling.

Abbreviations: *APC*, *Adenomatous polyposis coli* gene; BM, bone marrow; COX-2, cyclooxygenase 2; FAP, familial adenomatous polyposis; iMCs, immature myeloid cells; HNPCC, hereditary non-polyposis colon cancer; MMP, matrix metalloproteinase; NICD, Notch intracellular domain; NSAIDs, non-steroidal anti-inflammatory drugs; TGF-β, transforming growth factor-β.

Cancer remains at the highest mortality of disease deaths in most industrialized nations. Among the various cancer types, cancer of the digestive system is one of the commonest. Although the majority of lung cancer cases are caused by direct and/or indirect tobacco smoke, and therefore can be prevented by cessation of smoking, no clear direct causes are established for colon cancer, and few effective preventive measures are available. As most deaths by colon cancer are caused by their systemic dissemination and metastasis, the patients who are free of microscopic and macroscopic metastases can be cured by surgical resection of the primary tumours.

Rapid development in molecular biology in the last century has brought us rich knowledge in the mechanisms of carcinogenesis at the cellular level. They include the identification and characterization of oncogenes and tumour suppressor genes, as well as their roles in cancer formation by derailment from the normal cell physiology. However, application of such knowledge to effective treatment of human cancer is only beginning. Because cancer is a disease that affects the whole organism, it is imperative to use experimental animals to study the progression of cancer, including expansion of the primary tumour, invasion and metastasis. These 20 years or so, we have constructed multiple mouse models of colon cancer, analysed their mechanisms of tumourigenesis, expansion, invasion and metastasis (*1*–*3*). Based on these results, we have also invented new therapeutic strategies. This review describes the excerpts of such results centred on our own contributions to this field of cancer research.

Multi-step carcinogenesis by multiple, genetic and/or epigenetic changes

Through analyses of various human cancer types, it is suggested that several to less than 10 genetic/epigenetic changes are responsible for a particular type of cancer (*4*) (Fig. 1). These changes are generally thought to accumulate during a long time; often tens of years. For colon cancer, the earliest pathological change is the formation of an adenoma that is a benign tumour, but a premalignant lesion. Namely, some of the adenomas can progress to malignant (*i.e.* invasive and metastatic) colon cancer if left untreated. Human genetic studies on the familial adenomatous polyposis (FAP) patients led to the identification and characterization of the *APC* gene (*5*, *6*). Followed by these investigations, a linear chain of genetic/epigenetic events was proposed to be responsible for the development of the full-blown colon cancer (*7*). However, such a scheme still remains a working hypothesis, and the respective events and their order are not necessarily established, except that biallelic inactivation of the *APC* gene is the initiating event for the majority of cases. Especially, changes responsible for the invasion and metastasis are only beginning to be unravelled.

Mouse models for human FAP and chemoprevention studies for colon polyposis

We began with construction of a gene knockout mutant where the mouse homolog *Apc* of the human *APC* gene was artificially inactivated by embryonic gene manipulation ($Apc^{\Delta 716}$ mice) (*8*) (Fig. 2). In this mutant,

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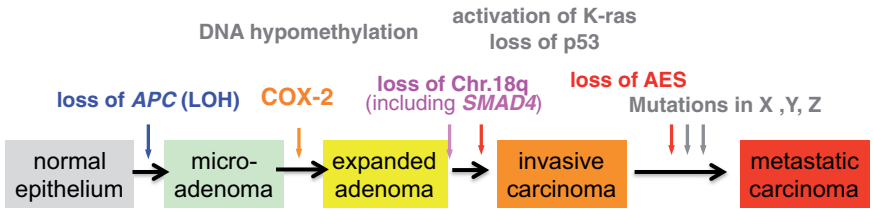

Fig. 1 A diagrammatic hypothesis of adenoma–carcinoma sequence in colon cancer formation and progression. Although DNA hypomethylation, activation of K-ras and loss of p53 are known to affect this sequence (*4*), the precise steps that are affected by such events are not discussed in this article, and printed in grey.

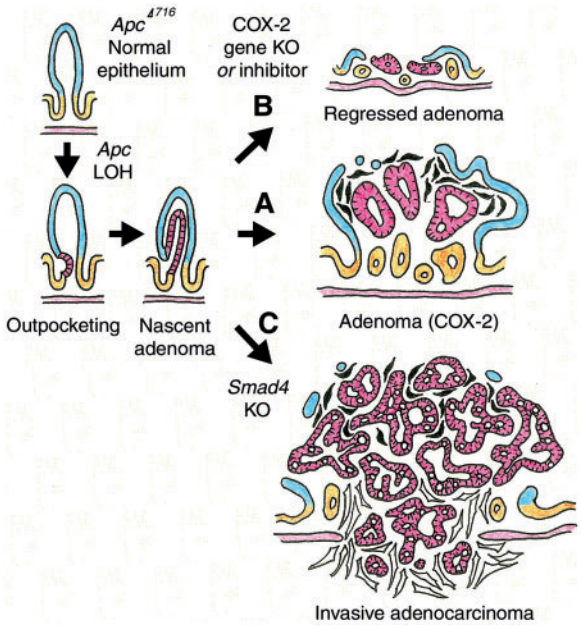

Fig. 2 Schematic drawing of mouse models of FAP and colon cancer invasion. From (*10*). The $Apc^{\Delta 716}$ mice carry a knockout mutation in one of the *Apc* alleles and develop numerous adenomatous polyps in the intestines after loss of the remaining *Apc* allele; LOH (*8*). In this model for human FAP, a nascent polyp is formed from an outpocketing pouch of the proliferation zone crypt epithelium, expanding into the inner (lacteal) side of a villus (*11*). In *A*, when such a nascent polyp expands, COX-2 is induced in the polyp stroma and plays an essential role in further growth. In *B*, disruption of the COX-2 gene (*Ptgs2*) or inhibition of COX-2 by a specific inhibitor suppresses the tumour growth (*12*). In *C*, on the other hand, introduction into the $Apc^{\Delta 716}$ mice of an additional knockout mutation of *Smad4*, a gene in the transforming growth factor β signalling, quickly converts the benign adenoma into a rapidly growing and invasive adenocarcinoma (*13*). *Blue*, normal villous epithelium; *yellow*, normal crypt epithelium; *pink*, tumour (adenoma or adenocarcinoma) epithelium; *black*, COX-2-expressing stromal cells; *white*, the activated fibroblasts; *purple*, the submucosal smooth muscle layer.

numerous adenomatous polyps are formed in the small intestine with only a few in the colon. This is in contrast to the human FAP where most polyps are formed in the colon. We later found that this pattern of polyp distribution shifts to that of human—mostly in the colon—when additional mutation in a homeobox gene *Cdx2* was introduced into the $Apc^{\Delta 716}$ mice (*9*).

Using the $Apc^{\Delta 716}$ mice, we investigated morphogenesis of the adenomatous polyps in the small intestine and found that an adenoma arose first in the proliferation zone above the crypt bottom, as an outpocketing to the proper mucosal space, rather than to the luminal side (11). For such nascent adenomas to expand beyond 0.5–2.0 mm in diameter, cyclooxygenase-2 (COX-2) plays a crucial role, because knocking out the COX-2 gene (*Ptgs2*) or dosing the mice with a COX-2 inhibitor suppresses polyp expansion (12). Importantly, expression of COX-2 is found only in the stromal fibroblasts at early stages of tumourigenesis, although the tumour epithelium also expresses COX-2 in more progressed human cancer.

Accordingly, we established the therapeutic strategy for treatment of FAP patients with COX-2 inhibitors. The key point in this therapy is to suppress the expansion of the adenomatous polyps in young FAP patients and prevent their carcinogenic progression, so that they can postpone the surgical resection of the colon (subtotal colectomy) often applied as a standard therapy. Although such an operation is safe, it necessitates the patient more than ten times of stool evacuation a day, often affecting the psychological development in young patients.

Based on these results, some pharmaceutical companies in the USA and Europe started large clinical trials of COX-2 inhibitors on sporadic colonic polyposis by which almost half of the senior citizens are affected. Unfortunately, these trials led to the side effects of cardiovascular accidents in the patients who were at higher risk for such events (14). Although the fraction of affected patients was rather small, actual number was seriously large simply because of the very large number of people who enrolled in the trials. This series of accidents led to the withdrawal of some, but not all, COX-2 inhibitors from the market, and similar side effects became obvious by some NSAIDs (non-specific COX inhibitors such as diclophenac) as well. However, this social tragedy was caused by an inappropriate clinical development that targeted senior patients of sporadic polyposis rather than the defects in the drug compounds. In fact, the use of COX-2 inhibitors such as celecoxib for young FAP patients remains as a useful therapeutic choice.

Bone marrow-derived cells assist local invasion of colon cancer

Colonic adenomas are benign tumours, although some of them can progress to malignant cancer if left untreated. These pre-malignant lesions can be removed easily during endoscopic examinations. The first

pathological sign of malignant changes in these tumours is their invasion into the submucosa. We constructed a compound mutant mouse strain that carried an additional knockout mutation in the *Smad4* gene that is involved in the TGF-β family signalling, in the background of the $Apc^{\Delta 716}$ polyposis mutant, and found that their intestinal tumours show marked local invasions (*13*). By detailed pathological and molecular analyses, we demonstrated that the tumour epithelium was producing a high level of chemokine CCL9 (corresponding to CCL15 in humans) that recruits bone marrow (BM)-derived immature myeloid cells (iMCs) to the tumour stroma as 'cap cells', and that they help tumour invasion by producing metalloproteinases MMP9 and MMP2 (*15*, *16*).

In human colon cancer, TGF-β signalling is often blocked in two alternative ways. In the majority of cases that is affected in the left side (to the sigmoid and rectum), 30–50% carries point mutations distributing widely in the coding region of *SMAD4* gene (*17*). In the hereditary non-polyposis colon cancer (HNPCC) cases that takes ~15% of the cases and often affected on the right side of the colon, the adenine cluster (A_{10}) in the coding region for the TGF-β type II receptor gene (*TGFBR2*) is affected by mismatch mutations (*e.g.* to A_9) that cannot be repaired by the defective host system (*18*). Because of the ease in PCR-based diagnosis, we analysed the tumour specimens from the latter (A_9 mutant specimens) and found infiltrations by CCR1-positive and MMP9-producing myeloid cells in ~1/3 of cases (*19*) (Fig. 3).

These results suggested that myeloid cells that were perceived generally to protect the mammalian body from cancer invasion in fact helped tumour invasion (*19*). Moreover, we identified the chemokine (CCL9 or CCL15) and its receptor (CCR1) that are responsible for this interaction between the tumour epithelium and the stromal microenvironment. Thus, colon cancer is soliciting the immune reaction to invade into the submucosa and beyond.

Bone marrow-derived cells assist metastatic expansion of colon cancer in the liver

Because cancer invasion into the submucosa appears to be the first step in the series of tissue reactions that leads to cancer metastasis, we hypothesized that a similar mechanism to that described above may be involved in the metastatic expansion (colonization) of colon cancer in the liver, the commonest organ of metastasis (*16*). Experimentally, we injected CMT93 colon cancer cells into the spleen of the syngeneic C57/BL6 mice, and allowed them to disseminate to the liver. Ten days later, we found that the injected cancer cells were surrounded by massive (5–10 times of the tumour cells) infiltrations of BM-derived cap cells. As anticipated, activation of the CCL9-CCR1 axis was essential for the expansion of the disseminated colon cancer cells, as evidenced by the lack of metastatic expansion when treated by an anti-CCL9 antibody or in the *Ccr1* gene knockout host mice. Interestingly, in the mutant hosts that lacked MMP9

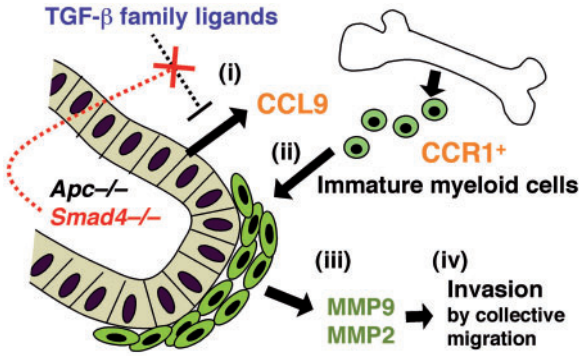

Fig. 3 Cap cells pilot invasion of colon cancer. From (*19*). Smad4-deficient tumour cells produce chemokine CCL9 and recruit the receptor-expressing cells that promote tumour invasion (*i*), the inactivation of the TGF-β family signalling within the tumour epithelium causes increased production of chemokine CCL9 (CCL15 in humans) because it is suppressed by TGF-β, activin-A and BMPs (*ii*). Increased levels of CCL9 (CCL15) recruit immature myeloid cells that carry the CCL9 receptor CCR1 from the blood to the tumour invasion front. These immature myeloid cells produce MMP9 and MMP2 (*iii*), and help the tumour epithelium to migrate and invade into the stroma *en masses* (*iv*).

or MMP2, the disseminated colon cancer cells could not expand despite that the BM-derived cap cells were recruited as in the wild-type hosts. These results indicate that the cap cells play critical roles in colon cancer progression not only in the local invasion, but also in the metastatic expansion after dissemination to the liver. To extend this research to clinical application, we further tested a CCR1 antagonist BL5923 developed by Novartis by dosing it in the liver dissemination model mice. We found that the CCR1 inhibitor reduced the number and size of the metastatic lesions in the host mice either injected with the mouse or human colon cancer cell lines (16).

It is widely known that proteases, especially metalloproteinases are involved in the invasion and metastasis of cancer. More than 10 inhibitors of metalloproteinases were tested in clinical trials by multiple pharmaceutical companies. Unfortunately, however, all trials failed due to severe side effects of headache, muscle and joint pain etc. In fact, it was very difficult to obtain blood concentrations high enough to be therapeutic. This failure is explained as that most metalloproteinases are heavily involved in the normal physiology of the human body in many tissues and organs, and therefore systemic inhibition of such important enzymes inevitably causes severe side effects (20).

Our results above indicate that the type of cells producing MMPs at early stages of colon cancer invasion is BM-derived rather than the tumour epithelium, and that tumour cells are simply recruiting the MMP-producing BM cells by secreting a specific chemokine (CCL9 or CCL15). Accordingly, we should be able to suppress invasion and metastasis of colon cancer if we can block the recruitment of MMP-producing BM cells, even if we do not inhibit MMPs systemically. Thus, we have coined this therapeutic strategy as 'cellular targeting therapy' in comparison with the 'molecular targeting therapy' (19).

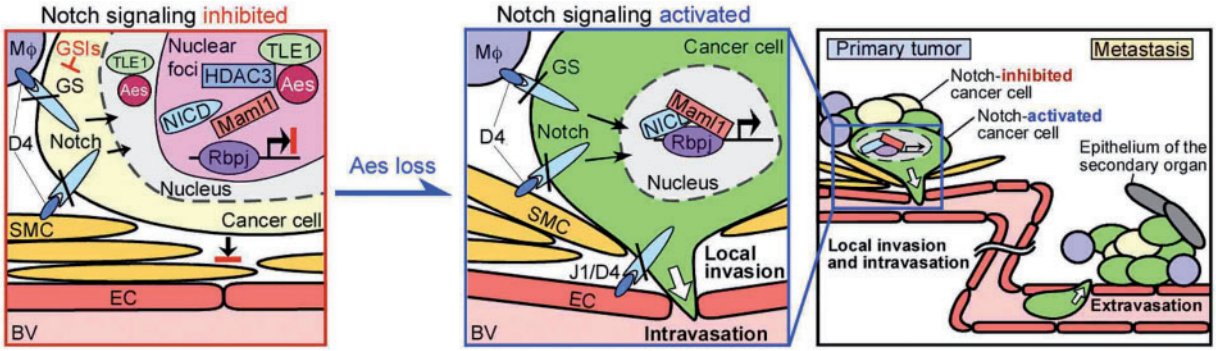

Fig. 4 A schematic drawing for colon cancer metastasis by loss of Aes expression. From (*21*). When Notch receptor expressed on a cancer cell is bound by Dll4 (D4) or Jagged1 (J1) ligand on adjoining macrophages (MØ), smooth muscle cells (SMC) or endothelial cells (EC), NICD is released by γ-secretase (GS) cleavage. If Aes is expressed in the tumour cell, however, the activated Notch receptor NICD is sequestered by Aes and TLE1 together with Rbpj, Maml, and HDAC3 in the nuclear foci (left). Once Aes is lost in the tumour cell, Notch signalling transcription by Rbpj is derepressed, stimulating its local invasion and intravasation into the blood vessel (BV, centre). In addition, Notch signalling also promotes extravasation of the cancer cell at the target organ, enhancing its metastasis (right). GSIs, GS inhibitors.

Mouse model for blood-borne metastasis of colon cancer to the liver and lungs

Using another mouse model of colon cancer metastasis, we have recently identified a novel metastasis suppressor gene *Aes* (21) (Fig. 4). Identification of this gene is based on our hypothesis that there must be endogenous metastasis suppressors, and loss of such a gene contributes to metastasis significantly. With a colon cancer cell line Colon26 derived from the Balb/c strain, we compared the primary tumours and metastatic lesions for expressed cDNA repertories by the microarray technology. Namely, we looked for genes whose expression is down-regulated in the metastases, among the transcription-related genes. Of approximately 20 such genes, we found that *Aes* (*Amino-terminal enhancer of split*) caused significant reduction of invasion in the Matrigel assay. We raised a specific antiserum to this gene product, and investigated Aes protein expression in human colon cancer specimens by immunohistochemistry. In the primary and metastatic tumour pairs from the same patients, we found that expression levels of Aes was significantly reduced in the metastases, suggesting that *AES* may be involved in metastatic suppression. We then investigated the role of *Aes*/*AES* on four major signalling pathways using specific reporter plasmids, and found that the protein suppresses the transcription of the Notch signalling through Rbpj transcription factor. On the other hand, it did not affect any of the Wnt, TGF-β or Hedgehog signalling pathways. Through microscopic analyses of the cultured cells, we also found that Aes protein forms insoluble nuclear foci together with Rbpj and other accessory transcription factors, and associates with the nuclear matrix.

Through immunohistochemical investigations of the Notch signalling ligands in colon cancer specimens, we found that Jagged1 ligand was expressed on the surface of blood vessels, whereas Delta-like4 ligand on macrophages and smooth muscle cells. On the other hand, tumour epithelium expressed abundant Notch receptors, suggesting that the Notch on the cancer cells can be activated as soon as they move out and make contacts with the surrounding tissues such as blood vessels, smooth muscle layers, or infiltrating macrophages. When the tumour cells express Aes, however, the activated Notch receptor (*i.e.* Notch intracellular domain; NICD) cannot bind Rbpj or activate transcription. If Aes expression is lost or reduced significantly, however, Notch signalling is activated through transcription of the Notch effecter genes. One of the clinically important outcomes of this signalling activation is the stimulation of intravasation and extravasation of the cancer cells through blood vessels, because *in vitro* assays of transendothelial migration (TEM) demonstrated dramatic increases in the number of colon cancer cells penetrating through the HUVEC (human umbilical vessel endothelial cell) layer. It is suggested in many types of cancer that tumour cells are disseminated from the primary lesion to a variety of distant organs during the surgical resection, and TEM contributes to these processes.

Perspectives

Since it was pointed out that cancer progression and inflammation are closely correlated each other by Virchow in 1863, the relationship was the subject of research for a long time with often confusing reports. It is worth re-evaluating the phenomena based on the more recent knowledge in the related fields (22). In addition to the cell types such as macrophages and granulocytes that participate in wound healing, some specific BM-derived cells as iMCs are recruited by chemokines and help invasion and metastasis of cancer. Notably, the recruitment of these cells coincides with cancer cell invasion and intravasiation around surgical excision of the primary tumours. Accordingly, it is conceivable that we can prevent further local invasion and metastatic colonization of the disseminated cancer cells by targeting CCR1 and/or Notch signalling as an adjuvant therapy immediately before and/or after the surgery of the primary tumours.

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Conflict of interest

None declared.

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